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CURRENT PROTOCOLS IN TOXICOLOGY

CURRENT PROTOCOLS

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NMR Analysis of Thiodiglycol Oxidation by Mammalian Alcohol Dehydrogenases

This unit describes a proton nuclear magnetic resonance (^{1}H NMR)-based methodology for investigation of thiodiglycol (TDG; Fig. 4.20.1A) transformation by human and equine alcohol dehydrogenases (ADH). An ultraviolet/visible (UV/vis) spectrophotometric method is also described in support of the NMR protocol. Its purpose is to initially indicate if TDG is actually a substrate for the various ADH isozymes before the more complicated NMR protocol is undertaken. Once verified spectrophotometrically that TDG is a substrate for a particular ADH isozyme, ^{1}H NMR is used to obtain structural and kinetic data on the products as they form. Although interest was established for the study of TDG metabolism with regard to how the hydrolysis of sulfur mustard (Fig. 4.20.1A) may contribute to its toxicity, the protocols in this unit can be applied as general methods for the metabolic study of different water-soluble xenobiotics using ADHs.

Access to NMR depends upon the facility in which an individual researcher works. Most large institutions have a centralized NMR facility where samples are submitted to an appointed scientist or technician. Because the personnel of the NMR facility have control of the instrumentation, they are responsible for acquiring data. This takes the operational aspects of running the NMR out of the hands of the researcher. Smaller research institutions usually have NMR as standard equipment, but they rely on the individual principal investigators and/or students to personally carry out their experiments as operators of the instrument. As such, this unit is being written in context of aiding an investigator who has to personally acquire and interpret the data. There are also multiple manufacturers of NMR spectrometers, each having a unique operational protocol. Therefore, this unit is also written from the standpoint that the specific operational steps in acquiring a spectrum are omitted. The user is referred to the equipment manufacturer's instruction manual.

UV/VISIBLE SPECTROPHOTOMETRIC ANALYSIS OF ADH ACTIVITY

The purpose of the UV/visible assay is to initially determine whether thiodiglycol (Fig. 4.20.1A) is a substrate for a particular isozyme of alcohol dehydrogenase before the more expensive and time-consuming ^{1}H NMR assay is performed. It is suitable for evaluating other potential substrates for ADH oxidation. This assay involves monitoring the optical density (OD) at 340 nm to detect the increasing concentration of reduced β-nicotinamide adenine dinucleotide (NADH) as shown generically in scheme 1 (Fig. 4.20.1B). Unlike the ^{1}H NMR assay (see Basic Protocol 2), no information regarding the structural identity of the metabolite(s) can be obtained. ADHs, as a class of enzymes, can catalyze dismutation reactions that are spectrophotometrically silent (Abeles and Lee, 1960; Henehan and Oppenheimer, 1993). Under these conditions no net production of NADH occurs, hence no increase in the absorbance at 340 nm. A compound would then appear to be inert to ADH-mediated oxidation. Even with this potential drawback, this spectophotometric assay is the quickest way to obtain reliable information regarding the ability of a purified ADH to oxidize a selected compound.

Materials

β-nicotinamide adenine dinucleotide (NAD) solution, warmed to 30°C
Thiodiglycol (see recipe)
Equine and human alcohol dehydrogenases (ADH; see recipe for equine liver ADH), on ice

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UV/visible spectrophotometer equipped with a constant temperature cuvette compartment (e.g., Beckman DU 70 or equivalent)
Standard 1.0-cm quartz cuvettes
30°C water bath
2.0-ml polypropylene microcetrifuge tubes (Eppendorf)

Set up spectrophotometer

1. Switch the UV/vis spectrophotometer from Idle to On.

2. Turn on the cuvette chamber temperature control and set the temperature to 30°C.

3. Turn on the visible lamp and allow it to warm up for at least 15 min.

4. Press Timed Drive and set the format to kinetic and OD, wavelength to 340 nm, time to 3.0 min, number of readings per minute to 10, and upper limit to 0.5 OD units using the Select key, the number pad, and the Enter key.

Figure 4.20.1 Structures and schemes described in this unit.
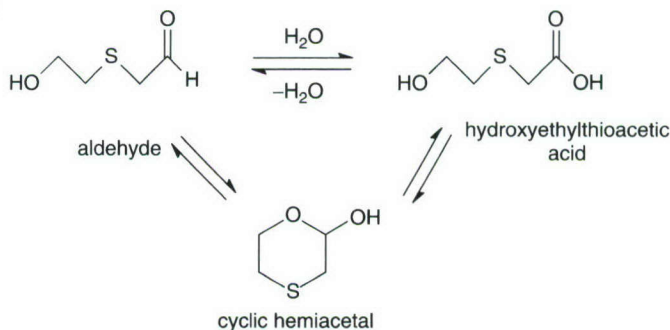
5. Turn on the printer and set the print quality indicator to Letter Quality.

6. After the instrument has warmed up, place a 1.0-cm cuvette containing 2.5 ml warm NAD solution into the reference cuvette holder. Close the lid and press Start.

   The instrument is now ready for an assay.

Prepare reaction mixture

7. Keep all reagent solutions except the enzyme (ADH) in a 30°C water bath.

8. Pipet 0.35 ml chilled ADH solution into a 2.0-ml microcentrifuge tube and warm to 30°C in the 30°C water bath.

9. When the ADH solution reaches 30°C, pipet 2.5 ml NAD solution and 0.25 ml ADH solution to a cuvette. Cap the cuvette and mix by gentle inversion. Warm 2 min in the 30°C waterbath.

10. Carry the temperature-equilibrated thiodiglycol solution and the partially filled cuvette from step 9 to the spectrophotometer.

11. Working quickly, pipet 0.25 ml thiodiglycol solution to the contents of the cuvette. Making sure the contents are bubble-free, invert gently to mix and place the cuvette in the spectrophotometer.

Run assay

12. Press Auto Zero, and when the blinking optical density indicator goes to zero, press Run to begin collection of data.

13. Collect the OD at 340 nm for 3 min at 10 points/min as programmed in step 4. Tabulate and print with the printer set on letter quality.

14. Make sure that the data sheets coming out of the printer are properly collated, stapled, and annotated so the assay conditions can be identified at a later date.

¹H NMR ANALYSIS OF THIODIGLYCOL OXIDATION BY ALCOHOL DEHYDROGENASE

Due to the cofactor requirement of ADH oxidations, this ¹H NMR protocol uses a cofactor regeneration system. The addition of lactate dehydrogenase and sodium pyruvate to the ADH reaction mixture allows for the use of catalytic amounts of the expensive NAD cofactor. The protocol also utilizes a presaturation technique to suppress the water signal from the solvent protons to allow for the visualization of the comparatively weak signals from the compounds of interest (Hore, 1989). Control spectra are initially generated utilizing the assay solution without the ADH. The enzyme is then added to the control solution and spectral data are collected over the course of several hours. From changes in the spectra relative to the control, it is possible to identify the terminal metabolite of thiodiglycol oxidation along with a transient species. In addition, it is possible to obtain kinetic data and the overall stoichiometry of the oxidation.

Because the procedures do not change when utilizing different sources of ADH, this protocol is written using the easily obtained, commercially available equine ADH. The cloned human isoforms of human ADHs are not commercially available and they are difficult to obtain. To carry out this assay using a different ADH, it is simply necessary to substitute the desired ADH for the equine variant in the protocol. Results obtained using a cloned human isoform are included in the discussion along with that of the equine enzyme. All interpretation of the spectra will be discussed in Anticipated Results.

Materials

0.1 M sodium phosphate buffer, pH 7.5/10% (v/v) D$_2$O (see recipe)
Lactate dehydrogenase (LDH from rabbit muscle; Sigma-Aldrich)
β-Nicotinamide adenine dinucleotide (NAD)
Sodium pyruvate
Thiodiglycol (Sigma-Aldrich)
1.6 U/mg equine or human alcohol dehydrogenase (ADH)

2.0- and 5.0-ml microcentrifuge tubes
10.0-ml centrifuge tubes
5.0-mm NMR tubes
600-MHz NMR spectrometer with variable temperature probe

NOTE: The individual steps in this protocol involve making and analyzing a multicomponent solution. Therefore, the solution recipes will not be repeated in Reagent and Solutions.

Prepare reagents and solutions

1. Chill the 0.1 M sodium phosphate buffer to 0°C.

2. Pre-weigh all reagents into individual containers prior to making the solutions.

   a. Weigh out 3.2 mg of commercially available lactate dehydrogenase from rabbit muscle into a 5.0-ml microcentrifuge tube. Cap tube and store on ice until needed.

   b. Weigh out 33.3 mg of NAD into a 5.0-ml microcentrifuge tube. Cap tube and store on ice until needed.

   c. Weigh out 275 mg of sodium pyruvate into a 5.0-ml microcentrifuge tube. Cap tube and store on ice until needed.

   d. Weigh out 122 mg of thiodiglycol into a 10.0-ml centrifuge tube. Cap tube and store on ice until needed.

   e. Weigh out 25 mg of commercially available equine alcohol dehydrogenase (1.6 U/mg solid) into a 2.0-ml microcentrifuge tube. Cap tube and store on ice until needed.

3. Make the control solution as follows:

   a. Dissolve the LDH in 2.3 ml of chilled 0.1 M sodium phosphate buffer, pH 7.5/10% D$_2$O. Set aside on ice.

   b. Dissolve the NAD in 5.0 ml of the chilled 0.1 M sodium phosphate buffer, pH 7.5/10% D$_2$O. Add 1.0 ml of this solution to the LDH solution prepared in step 2a. Set the NAD plus LDH solution aside on ice.

   c. Dissolve the sodium pyruvate in 5.0 ml of chilled 0.1 M sodium phosphate buffer, pH 7.5/10% D$_2$O. Add 1.0 ml of this solution to the NAD/LDH solution prepared in step 3b. Set aside on ice.

   d. Dissolve the thiodiglycol in 10.0 ml of chilled 0.1 M sodium phosphate buffer, pH 7.5/10% D$_2$O. Add 0.5 ml of this solution to the NAD/LDH/pyruvate solution prepared in step 3c. Set this solution aside on ice.

   The above steps result in a chilled solution of LDH/NAD/pyruvate/thiodiglycol in sodium phosphate buffer, pH 7.5/10% D$_2$O with a total volume of 4.8 ml.

4. Transfer a 0.48-ml aliquot of the LDH/NAD/pyruvate/thidiglycol solution to a standard 5-mm NMR tube.

   The contents in this NMR tube will be 248 U LDH/2 mM NAD/100 mM sodium pyruvate/10 mM thiodiglycol.
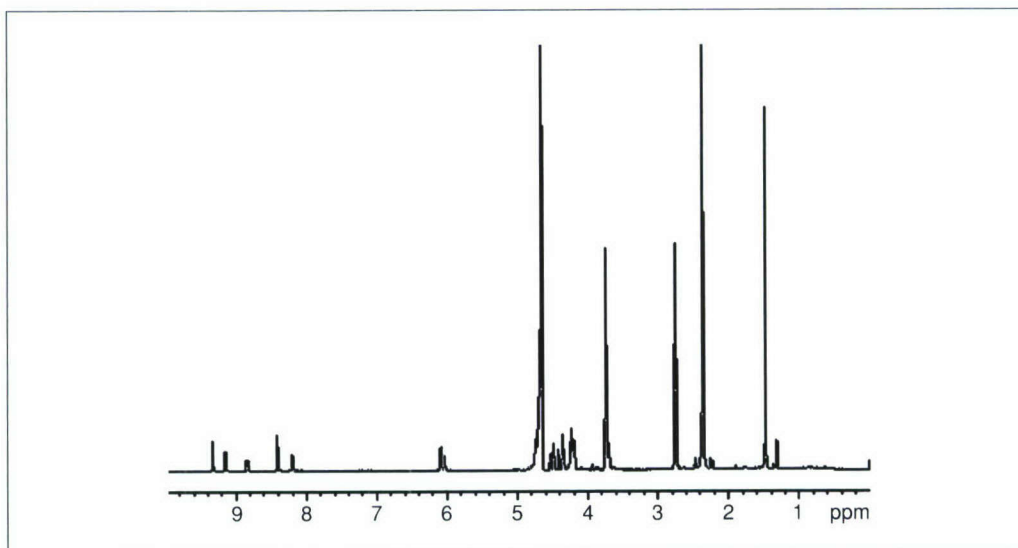
Figure 4.20.2 Control spectrum illustrating chemical shifts of the various components of the assay mixture prior to the addition of ADH.

5. Utilizing the procedure specified by the NMR manufacturer's instructions, set the probe temperature to 37°C. Insert the NMR tube containing the control solution into the instrument and allow the contents to equilibrate over 15 min to 37°C.

6. Collect a ^{1}H NMR spectrum on this control solution utilizing the procedure specified by the instrument manufacturer's instructions. Acquire 64 scans with a spectral width of 10,000 Hz using 32,000 data points. Utilize a 3.9-sec post-acquisition delay to allow for full relaxation of the resonances.

   A spectrum taken on this control sample is shown in Figure 4.20.2.

Collect data on ADH-mediated oxidation

7. Remove the NMR tube containing the control solution from the spectrometer.

8. Dissolve the equine ADH previously weighed out in step 2e in 1.0 ml of chilled 0.1 M sodium phosphate buffer, pH 7.5/10% D_2O.

9. Immediately add a 20.0-μl aliquot of this solution to the NMR tube containing the control mixture.

10. Using the parameters in step 6, acquire a spectrum every 8 min for the first 30 spectra and then every 30 min out to 10.5 hr.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; *for suppliers, see* **SUPPLIERS APPENDIX**.

Equine liver alcohol dehydrogenase solution, 1 mg/ml

   Weigh out 10.0 mg of equine liver ADH (1.6 U/mg; Sigma-Aldrich) and transfer to a clean 10.0-ml volumetric flask. Dilute with shaking to the 10.0-ml mark with Tris-maleate buffer (see recipe). Prepare fresh daily and keep on ice.

NAD solution, 5 mM

   Weigh out 33.3 mg of β-nicotinamide adenine dinucleotide (NAD; Sigma-Aldrich) and transfer to a 10.0-ml volumetric flask. Dilute with 5.0 mM Tris-maleate buffer (see recipe) with shaking to the 10.0-ml mark. Prepare fresh daily and keep on ice.

Techniques for Analysis of Chemical Biotransformation

4.20.5

Sodium phosphate buffer, pH 7.5, 0.1 M

Make stock solutions of 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 in 500-ml volumetric flasks. Combine 405 ml of 0.2 M K_2HPO_4 solution and 95 ml of 0.2 M KH_2PO_4 solution in a 1.0-liter volumetric flask. With mixing, add 100 ml of 10% (v/v) D_2O and then dilute to the 1.0-liter mark with distilled water. Transfer this solution from the volumetric flask to a clean 1000-ml beaker containing a Teflon stir bar. With stirring, adjust the pH to 7.5 with 20% (w/v) aqueous KOH. Transfer the buffer solution to a clean screw-cap jar for storage. Store up to 2 months at 4°C.

Thiodiglycol solution, 200 mM

To a clean 100-ml volumetric flask, add 0.85 g NaCl and 2.44 g thiodiglycol. Dilute to the 100-ml mark with distilled water and shake. Prepare fresh daily and keep on ice.

Tris-maleate buffer, pH 7.6, 20 mM

Weigh out 4.74 g of Tris-maleate (TRIZMA maleate) and transfer to a 1.0-liter volumetric flask. Fill the flask approximately three-quarters full with distilled water. Swirl the flask until the Tris-maleate is dissolved. Dilute the solution with distilled water to the 1.0-liter mark. Transfer this solution from the volumetric flask to a clean 1000-ml beaker containing a Teflon stir bar. With stirring, adjust the pH to 7.6 with solid maleic acid or hydrochloric acid. Transfer the buffer solution to a clean screw-cap jar for storage. Store up to 2 months at 4°C.

COMMENTARY

Background Information

Elucidating the metabolism of xenobiotics at the molecular level is crucial to understanding their observed toxicity. Of the various spectroscopic techniques now available to aid in these studies, NMR is the most valuable. It is a sensitive technique that provides the least ambiguous data with regard to molecular structure. In addition, it has the capability of providing kinetic data, determining stereo- and regiochemical purities; it is flexible enough to carry out both in vitro and in vivo studies; and it does not rely on the presence of chromophores. NMR assays also have other advantages in that one can detect transient species and minor products that would otherwise not be seen in large-scale enzymatic batch processes and/or with other forms of spectroscopy.

The ^1H NMR method presented in this unit is a detailed continuation of the authors' earlier work investigating the oxidation of thiodiglycol with a variety of different alcohol dehydrogenases (Brimfield et al., 1998; Dudley et al., 2000). Until the NMR assay was employed, any information gathered on the nature of the oxidation and the identity of the metabolites was speculative. A priori, one might predict that the product of thiodiglycol oxidation by ADH would be the aldehyde, as generically illustrated in scheme 2 (Fig. 4.20.1C).

However, the authors could not exclude the possibility of a second oxidation leading to the formation of 2-hydroxyethylthioacetic acid via a dismutative mechanism. Complicating things further is the possibility for the aldehyde to exist in an equilibrium mixture with its hydrate and its cyclic hemiacetal. This would be due to the aqueous conditions under which the ADH assays are typically run (scheme 3; Fig. 4.20.1D).

It became evident that ^1H NMR was the only spectroscopic method that would give detailed insight into the nature of thiodiglycol oxidation. The drawbacks of other spectroscopic methods become apparent when looking at the reaction conditions and the characteristics of the substrate and the potential intermediates and metabolites. They lack the chromophores necessary for UV/vis analysis. The aqueous conditions and relatively low concentrations prohibit infrared analysis. Mass spectral analysis, although very sensitive, only gives partial structural data in addition to a molecular weight. For example, it would be hard to differentiate between the aldehyde and the cyclic hemiacetal due to the fact that they have identical molecular weights and would be expected to fragment in a similar manner.

Critical Parameters and Troubleshooting

Choice of buffer

There is some controversy on the use of Tris buffers in ADH assays. Under certain conditions, an aldehyde generated by the ADH-mediated oxidation of an alcohol can react with the Tris to form a Schiff base (Trivic et al., 1998). This could lead to incorrect reaction stoichiometry if measuring NADH production spectrophotometrically or if assuming aldehyde dismutation is taking place.

Shimming the NMR

For researchers who are new to acquiring their own NMR data, the most important operational aspect involves shimming the instrument prior to the actual data collection. When one shims the instrument, they are optimizing the homogeneity of the magnetic fields. The exact procedure for shimming an instrument is dependent upon the manufacturer, but can be carried out either manually or automatically. Improper shimming can lead to reduced resolution, reduced sensitivity, and artifacts such as peak splitting, with the result being the possibility of data misinterpretation. Figure 4.20.3 is a spectrum that was obtained utilizing the same procedures delineated in Basic Protocol 2 using equine ADH. However, it was intentionally run with poor shimming of the instrument to illustrate the effect on the data. It can be seen that the singlet corresponding to the pyruvate now appears as an asymmetrical doublet, and that peak broadening occurs with loss of resolution in the splitting patterns. Utilizing the poorly shimmed spectra, one may not correctly identify metabolites due to the apparent peak splitting and coupling constants (distance between peaks).
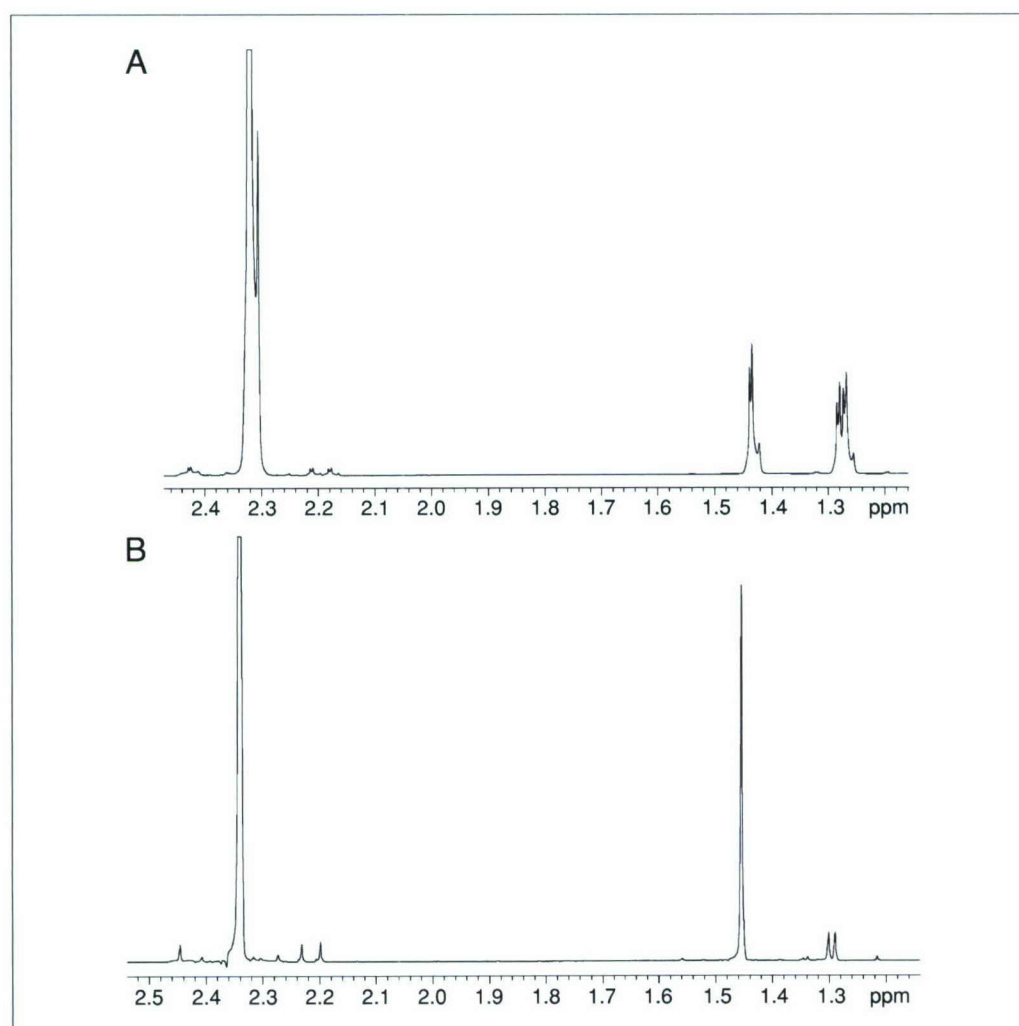

Figure 4.20.3 Comparison of sample spectra obtained with poor shimming of the NMR (**A**) with that of the same sample obtained with proper shimming (**B**).
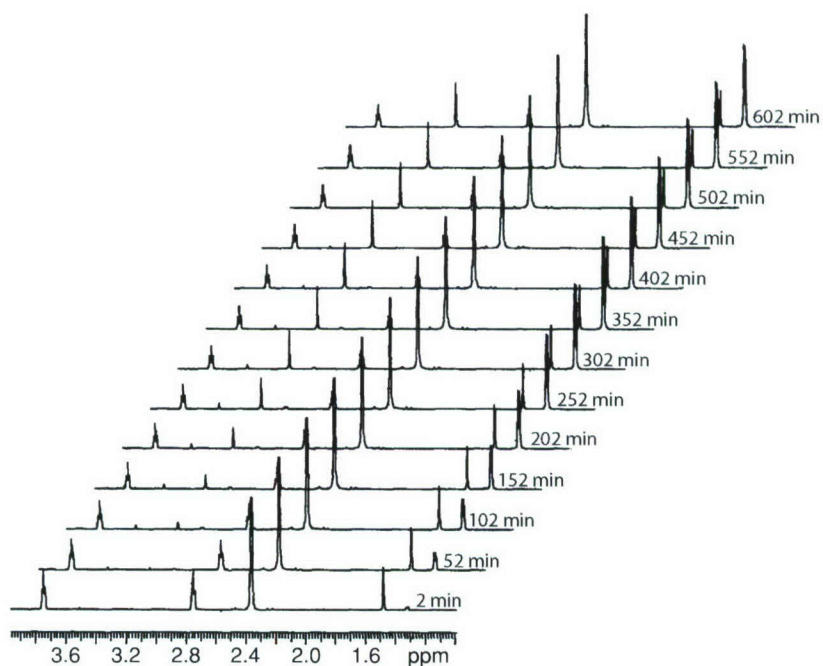
Figure 4.20.4 The sequential ^1H NMR spectrum of TDG metabolism by equine ADH from 2 to 602 min showing the evolution of lactate at 1.24 ppm and 2-hydroxyethylthioacetic acid at 3.23 ppm. There are 50 min separating each spectrum.

Figure 4.20.5 The sequential ^1H NMR spectrum of TDG metabolism by human α ADH from 2 to 402 min showing the evolution of lactate at 1.24 ppm and 2-hydroxyethylthioacetic acid at 3.23 ppm. There are 50 min separating each spectrum.
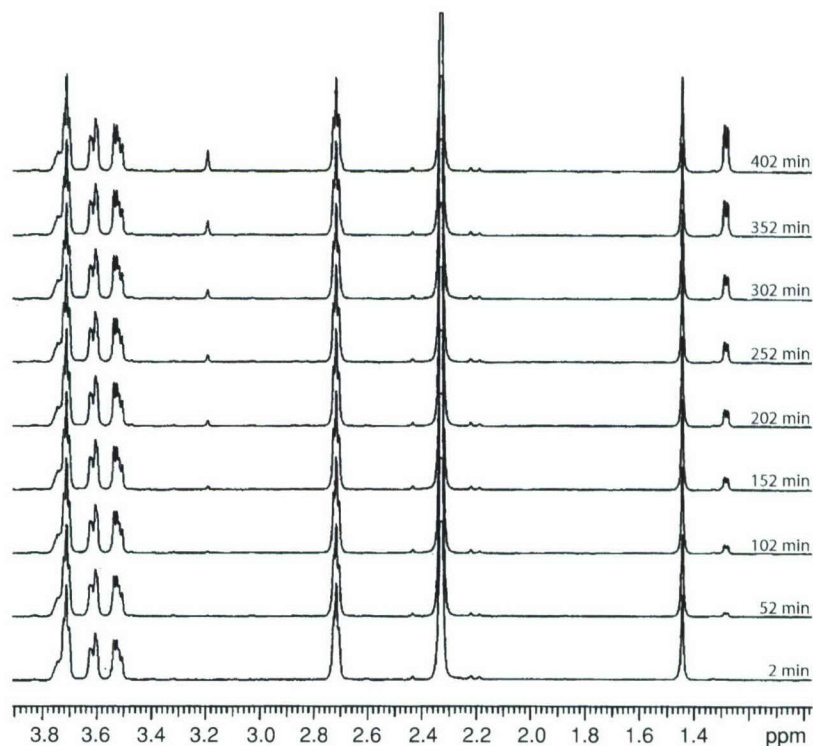
Choice of substrate

Choice of substrate is important if one wants to obtain kinetic data on the oxidation or simply monitor the disappearance of the substrate over time. Due to the presence of the NAD regeneration system, it is recommended to take a ^{1}H NMR spectrum of the substrate before it is analyzed using Basic Protocol 2 to verify that the signals due to the pyruvate, hydrated pyruvate, NAD, and lactate do not interfere with those of the substrate. Also, the substrates utilized in a protocol such as this need to have some degree of water solubility.

Anticipated Results

The primary focus is on interpretation of the ^{1}H NMR data with the UV/vis data included in a supporting role. Once data is collected from the instrument, the emphasis is on the correct interpretation of the spectrum, especially when it represents a mixture of different compounds. A good text for the novice is that of Silverstein et al. (2005). It provides an excellent foundation for the interpretation of NMR data.

As mentioned previously, the NMR protocol can be utilized as a general method for the study of water-soluble compounds and their oxidation by ADHs. Regardless of the substrate studied or the ADH isoform used, certain features will always be present in the NMR spectra. These features are illustrated in Figure 4.20.2, and are due the cofactor regeneration system in the control solution. The signals downfield of 6.0 ppm represent the catalytic amount of NAD. The singlet at 2.8 ppm is due to the methyl group on the pyruvate, which is the substrate for the LDH. Because a carbonyl can exist as a hydrate in aqueous media, the singlet seen at ~1.5 ppm is due to the hydrated form of pyruvate. The large signal at 4.8 ppm is the residual water peak that has been greatly reduced in intensity by the water suppression algorithm present with modern NMR
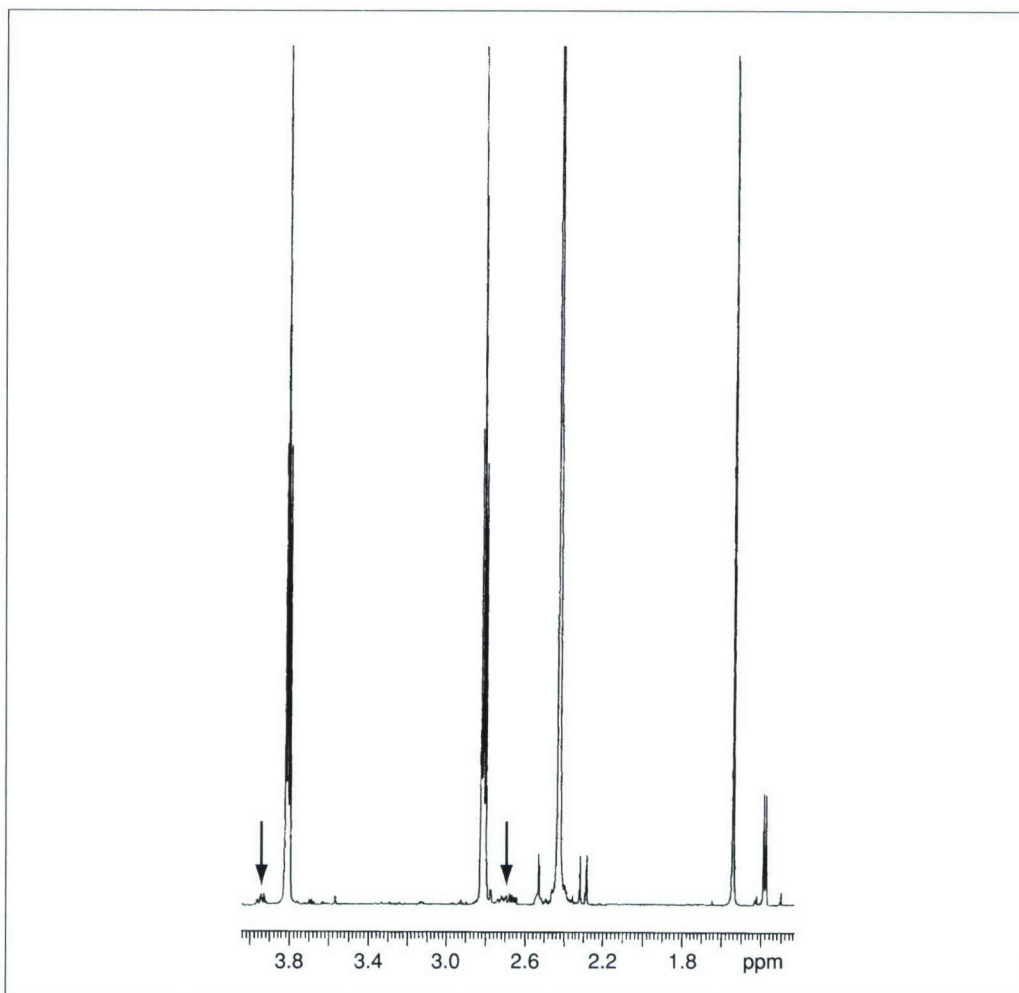

Figure 4.20.6 Expanded portion of the ^{1}H NMR generated using equine ADH prior to observation of the 2-hydroxyethylthioacetic acid metabolite. The arrows represent signals due to the low concentration of the transient intermediate 1,4-oxathian-2-ol.
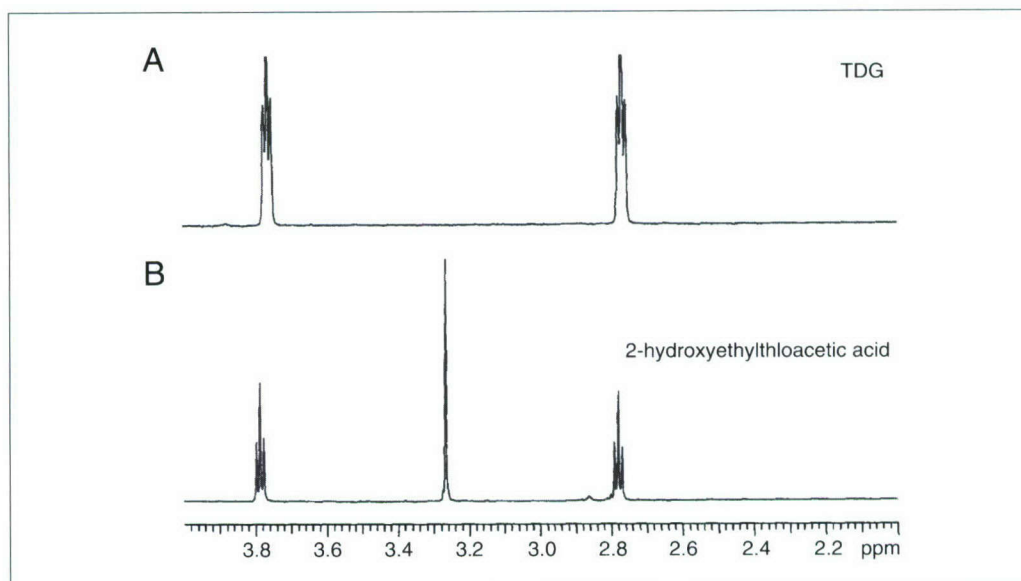
Figure 4.20.7 ¹H NMR data showing the direct comparison of TDG (**A**) with synthetically derived 2-hydroxyethylthioacetic acid (**B**). The singlet at 3.23 ppm is due to the protons α to the carbonyl.
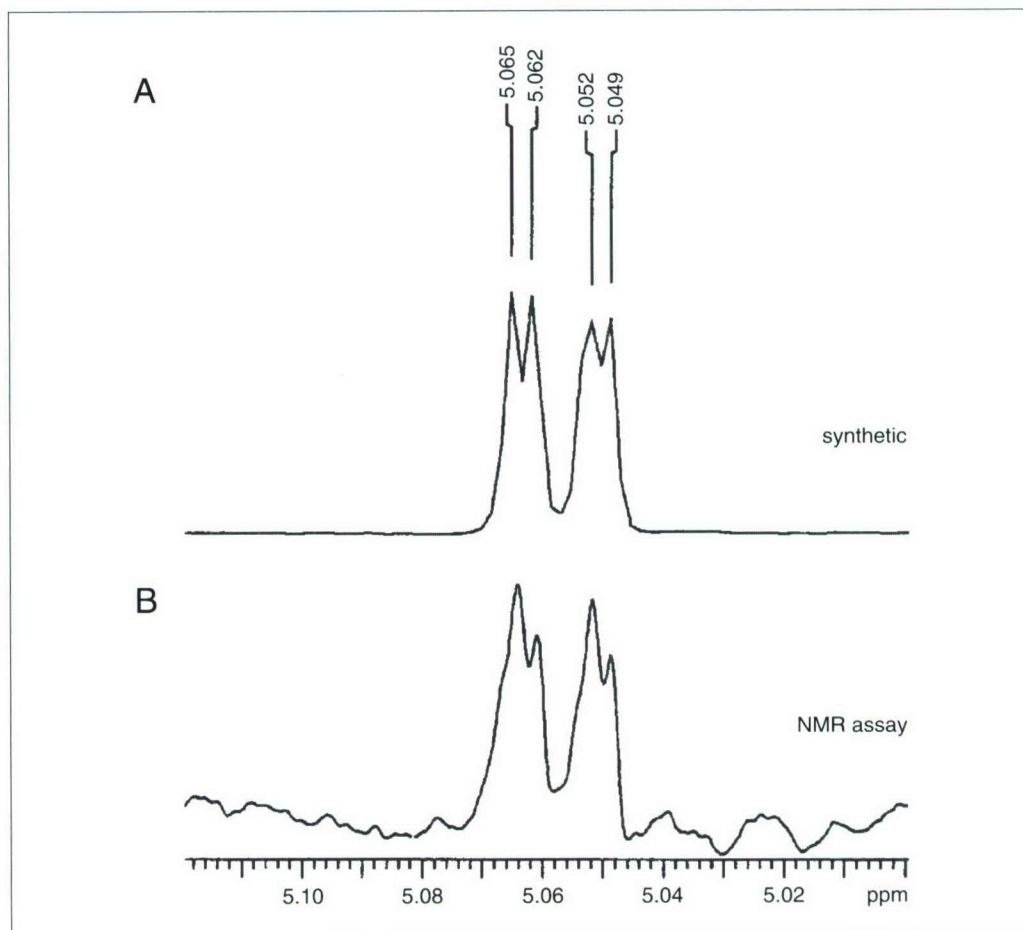

Figure 4.20.8 ¹H NMR data showing the direct comparison of synthetically derived 2-hydroxyethylthioacetic acid (**A**) to that of the ADH assay mixture (**B**). This absorption is due to the anomeric proton as indicated.

spectrometers. Also in the control reaction is the presence of two triplets at 2.78 and 3.78 ppm. These resonances correspond to the protons on thiodiglycol.

Once the ADH of choice is added to the control solution, one should observe a slow change in the sprectra over time. Illustrated in Figures 4.20.4 and 4.20.5 are composites of several spectra that were taken over the course of several hours utilizing the equine ADH and the human α isoform. The change in the spectra can be clearly noted. The figures include expanded portions of the spectra that contain the important changes. The doublet that appears at 1.32 ppm is due to the lactate that forms upon LDH reduction of the pyruvate. The other signal due to lactate is a quartet at 4.05 ppm (not shown). Also observed in these spectra is the gradual appearance of a singlet at 3.23 ppm. Its presence as a singlet indicates that the carbon containing these protons was an isolated spin system in that all neighboring atoms lacked protons. Examination of the potential metabolites in schemes 2 and 3 (Fig. 4.20.1C,D) shows that only one compound, 2-hydroxyethylthioacetic acid, contained an isolated spin system as represented by the protons α to the carbonyl. The

protons on the 2-hydroxyethyl moiety, being isoelectronic with those of thiodiglycol, would remain buried under its resonances at 2.78 and 3.78 ppm.

At this point, the only apparent compound formed by the ADH-mediated oxidation of thiodiglycol was tentatively identified as 2-hydroxyethylthioacetic acid. However, a closer look at the initial NMR data prior to observing the formation of 2-hydroxyethylthioacetic acid, revealed what appeared to be baseline noise in the spectrum as indicated by the arrows in Figure 4.20.6. It was observed that this "noise" disappeared over the course of the assay. This suggested that what was being observed corresponded to the formation and subsequent disappearance of a transient intermediate. Expansion of the regions indicated by the arrows in Figure 4.20.6 indicates that this transient intermediate could possibly be the cyclic hemiacetal in scheme 3 (Fig. 4.20.1D). This datum is interesting because it shows that thiodiglycol undergoes a two-step oxidation by equine ADH, whereby the initial product dissociates from the active site prior to the second oxidation. It also indicates that the second oxidation is kinetically quicker that that of the first. This is information
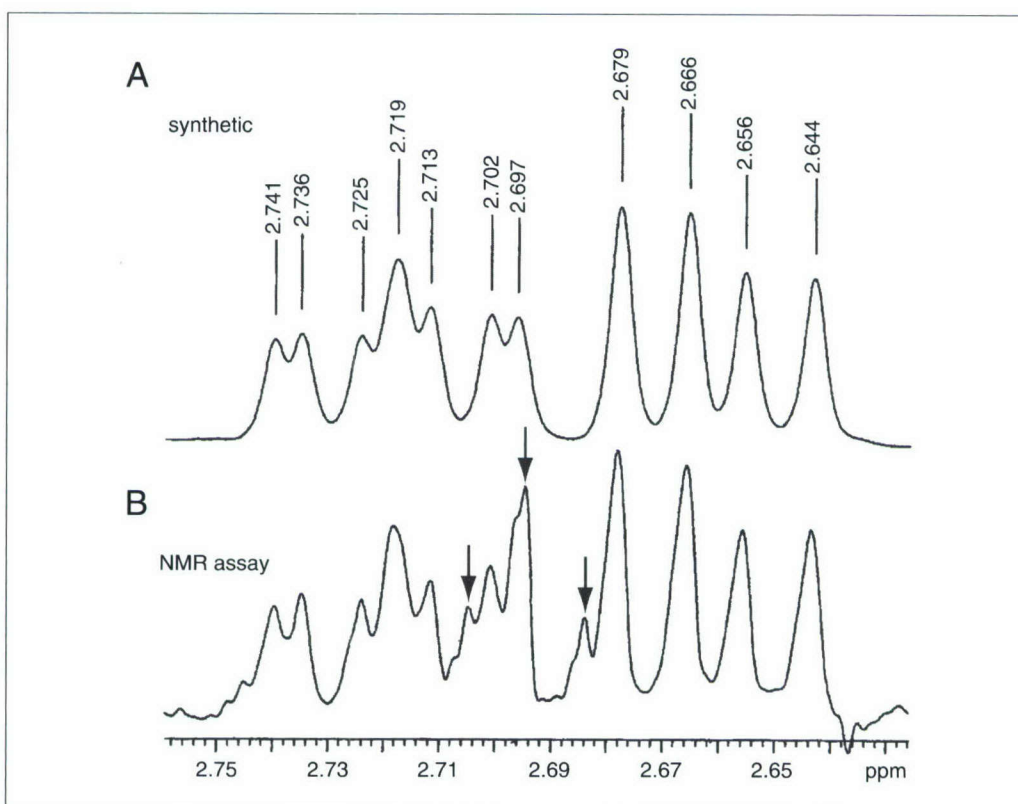

Figure 4.20.9 ¹H NMR data showing the direct comparison of synthetically derived 2-hydroxyethylthioacetic acid (**A**) to that of the ADH assay mixture (**B**). The arrows indicate a satellite peak of TDG caused by proton coupling with small amounts of naturally occurring carbon-13.

Techniques for
Analysis of
Chemical
Biotransformation

4.20.11

Current Protocols in Toxicology

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that could only be gained through the use of ¹H NMR spectroscopy.

To conclusively identify the structures of the metabolites, the authors then turned to independent synthesis. Although beyond the scope of this unit, the ability to independently synthesize suspected metabolites in the laboratory can greatly facilitate metabolism studies in that it allows direct comparison of the ¹H NMR assay results with that of a synthesized standard. Because the authors suspected the major metabolite to be the acid, it was synthesized in two steps using standard chemistry (Black et al., 1993). Illustrated in Figure 4.20.7 are portions of two ¹H NMR spectra representing that of thiodiglycol, and that of the independently synthesized 2-hydroxyethylthioacetic acid. Based on this comparison, the major oxidative metabolite of TDG was conclusively identified to be 2-hydroxyethylthioacetic acid when both equine and human α ADHs were used. By substi-

tuting the acid (hydroxyethylthioacetic acid) for thiodiglycol in the UV/vis assay (see Basic Protocol 1), the authors were able to determine that this compound was the terminal metabolite. No NADH formation was observed, illustrating that the 2-hydroxyethyl moiety is not oxidized by the ADH.

The same strategy was used to confirm the identity of the transient intermediate (Figure 4.20.6). Oxidation of TDG utilizing a Cr(VI)-pyridine reagent according to the protocol of Corey and Schmidt (1975), yielded the cyclic hemiacetal (scheme 3; Fig. 4.20.1D). Figures 4.20.8 and 4.20.9 are direct comparisons of the synthetically derived material and that of the expanded region of the ¹H NMR assay. From this direct comparison, it was conclusively established that the transient intermediate observed was due to initial formation of 1,4-oxathian-2-ol by ADH-mediated oxidation of TDG. It also demonstrated that the free aldehyde and its hydrated analog do not
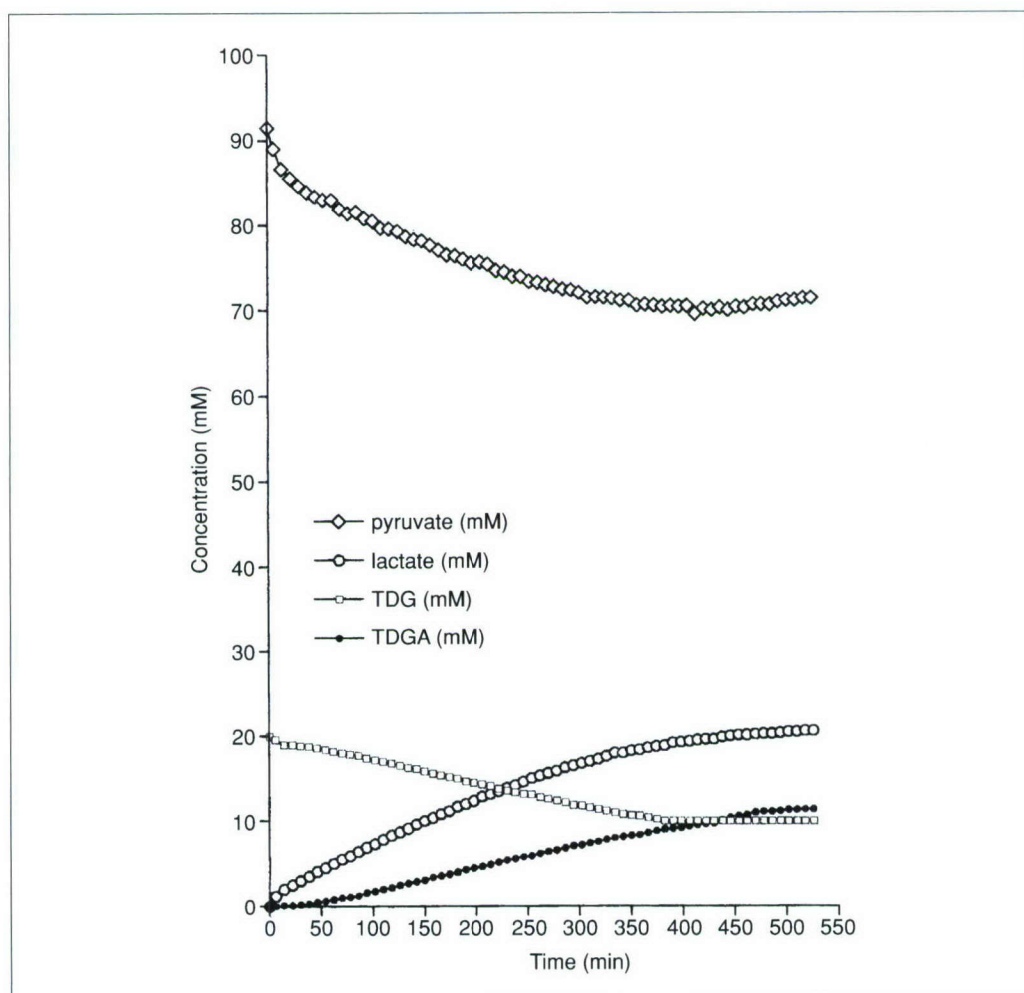

Figure 4.20.10 Kinetic plot generated from sequential spectra based on peak integrations illustrating the stoichiometric relationship between disappearance of TDG and pyruvate, and formation of lactate and metabolite.

exist in appreciable quantities under the assay conditions.

Lastly, as illustrated in Figure 4.20.10, ^{1}H NMR can be used to determine the overall stoichiometry of a reaction in support of a proposed mechanism. The area of a peak in a ^{1}H NMR spectrum is directly proportional the amount of material. Although it may appear that the relative sizes of the pyruvate and TDG resonances do not change (Figs. 4.20.4 and 4.20.5) during the course of the assay because of the high starting concentration, graphing the integrated data of these resonances showed a reduction in their concentrations is consistent with an increase in lactate and metabolite production. From this data it was determined that 2 moles of NAD were required per mole of TDG oxidized. This is mechanistically consistent with the formation of the metabolite.

Time Considerations

Data generation represented in these protocols represent more than a typical full day's work, especially if run back to back. The ADH enzymes are typically active in the NMR tube for 8 to 12 hr. However, most modern NMR instruments have the capability of automatically generating and saving spectra at preprogrammed intervals, giving an investigator the afternoon to accomplish other tasks once the reagents and solutions have been prepared and the UV/vis assay carried out.

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Techniques for
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